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ARTICLE

Indirect organogenesis from various explants of *Hildegardia populifolia* (Roxb.) Schott & Endl. – A threatened tree species from Eastern Ghats of Tamil Nadu, India

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In vitro

Abstract *Hildegardia* species are an important resource for fiber industry. This investigation was conducted to develop a plant regeneration protocol for *Hildegardia populifolia* (Roxb.) Schott & Endl. via indirect organogenesis Callus was obtained from leaf, internode and petiole explants, among these explants internode explant gave best result on MS medium supplemented with different concentrations of 2,4-Dichlorophenoxy acetic acid (2,4-D). The highest percentage (100%) of regeneration was obtained with benzyladenine (BA) (2.0 mg/l) + indole-3-acetic acid (IAA) (0.1 mg/l) + glutamine (25 mg/l) + thidiazuron (TDZ) (0.5 mg/l) from internode explants. Shootlets were highly rooted on MS medium supplemented with 3.0 mg/l indole-3-butyric acid (IBA). *In vitro* rooted seedlings were successfully acclimatized. This *in vitro* regeneration system will facilitate further development of reliable procedures for this genus.

1. Introduction

Hildegardia populifolia (Roxb.) Schott & Endl. is a medium sized deciduous tree of the family Sterculiaceae confined to tropical forests of Tamil Nadu and Andhra Pradesh in India. A narrow endemic [2], *H. populifolia* was known to be represented by a sole surviving population comprising about 20 trees in Kalrayan Hills of Tamil Nadu [1]. This species is under threat due to factors not apparent at present. But it is assumed that anthropogenic interference, habitat losses and other intrinsic and extrinsic factors might have been the reason for their poor regeneration ability and low seed viability [21]. According to IUCN [14] list of threatened species, *H. populifolia* is critically endangered. Sarcar and Sarcar [29] located 13

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mature trees and 11 young natural regenerations of this species in dry mixed deciduous forests of Parigam Reserved Forests in Kalrayan Hills of Kallakurichi Forest Division, Tamil Nadu and they assessed its status as threatened. This endemic species was evaluated in its entire range and placed under IUCN category, critically endangered [27]. This tree is easily recognizable by its pale green bark. The fiber extracted from the bark is used for domestic purposes and leaf extract is known to have healing properties [28].

In vitro regeneration is an efficient means of *ex situ* conservation of plant diversity [9,15,5] because with this technology many endangered species can be quickly propagated and preserved using minimal of plant material. Moreover this technique has the unique advantage of propagating the desired taxon, independent of season, reproducing barriers and germination hurdles. *H. populifolia* is ideal candidate for *in vitro* regeneration with its intrinsic poor seed viability and regeneration capabilities. Available literature [3,16,8] reveals that an *in vitro* protocol for this vulnerable tree has been carried out through distinct *in vitro* regeneration approaches i.e. indirect organogenesis. Protocols for organogenesis and somatic embryogenesis of this plant from seedling derived axillary meristem and mature tree derived nodal meristem are standardized [3]. The present study describes successful plant regeneration via adventitious indirect organogenesis from internode, petiole and leaf segments of *H. populifolia*.

2. Materials and methods

2.1. Seed germination and explant preparation

Seeds of *H. populifolia* were collected on November 2004 from Kalrayan Hills which 300–600 m, Eastern Ghats of Tamil Nadu. Chemical scarification was carried out by soaking the seeds in concentrated H_2SO_4 (98%) for 5, 15, 30, 45, 60 and 90 min and subsequently rinsing with tap water for 30 min. The scarified seeds were then disinfected with 70% ethyl alcohol for 5 min and followed by aqueous solution of 0.1% mercuric chloride for 10 min. Then the seeds were rinsed 4–5 times with sterile distilled water. Sterilized seeds were aseptically inoculated in MS medium supplemented with GA_3 (1.0 mg/l) and cotton soaked with sterile water. The inoculated seeds were incubated under optimal culture condition. The *in vitro* raised seedlings were transferred to earthen pots containing soil and sand (1:1) maintained in the garden. The explants were collected from 45 day old seedlings and cut into 10–15 mm fragment as explants.

2.2. Media and culture condition

The basic culture medium (BCM) consists of Murashige and Skoog [20]. MS medium was fortified with 30 g/l sucrose (Himedia, India) and gelled with 0.8% agar (Himedia, India), and the pH of the medium was adjusted to 5.7 ± 0.2 with 0.1 N NaOH or 0.1 N HCl after addition of the growth regulators. The medium was dispensed in culture tube and autoclaved at 121 °C, for 30 min. All the cultures were maintained in a sterilized culture room at 26 ± 2 °C, under 16 h photoperiods provided by cool white fluorescent light (60 $\mu mol^{-2} s^{-1}$) and with 55–60% relative humidity. The cultures were subcultured on the fresh medium after 15–20 days.

2.3. Callus initiation

Explants including internode, (1 cm in length), petiole (0.5 cm in length), and leaf (0.5 × 0.5 cm) were excised from 45 day old *in vitro* germinated seedlings and placed horizontally on MS medium. In this experiment, the effects of cytokinins and auxins, both separately and in combination were studied on callus initiation and its proliferation. Auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D), indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA) (0.1, 0.5, 1.0, 2.0 and 3.0 mg/l), and cytokinins benzyladenine (BA) and (kinetin) Kin (0.5, 1.0, 2.0 and 3.0 mg/l) either alone or in combinations of IAA + BA, IAA + Kin, and Kin + BA. For further callus proliferation supplements such as adenine, adenine sulfate, glutamine and sodium citrate (10, 25 and 50 mg/l) were used. These culture conditions were used in all the experiments mentioned below unless stated. Data of frequency (%) of callus formation and its fresh weight were recorded after 45 days of culture.

2.4. Shoot organogenesis from callus

Well-established hard and compact callus (~0.5 g fresh weight) were grown on MS medium supplemented with BA (2.0 mg/l) + IAA (0.1 mg/l) + glutamine (25 mg/l) for shoot organogenesis. Calli were transferred to shoot organogenesis media, consisting of MS basal media supplemented with TDZ (0.1, 0.5, 1.0, 2.0 and 3.0 mg/l) and activated charcoal (5, 10 and 25 mg/l), in combinations of TDZ at 0.5 mg/l. Cultures were transferred on fresh media after 20th day of inoculation. The percent of shoot organogenesis from callus and average number of shoots per inoculum were recorded on the 35thday after transferring the callus on shoot organogenesis media.

2.5. Formation of adventitious root

Regenerated shoots (2–4 cm long) obtained from micropropagated plantlets were cultured on MS basal medium fortified with either of IBA, NAA, or IAA (0.1, 0.5, 1.0, 2.0, and 3.0 mg/l) for adventitious rooting. Data were recorded on percentage of rooting, number, and length of the roots after 4 weeks of transfer onto the rooting media.

2.6. Hardening of regenerated plants

Rooted plantlets were removed from the medium and washed in sterile distilled water to remove all the traces of agar and basal callus. The plantlets were then transferring to plastic pots (5–6 cm diameter) containing garden soil mixed of vermiculite and sand (2:1:1). The plastic pots were covered with polyethylene bags to maintain the relative humidity of about 70–80%. These pots were maintained at 25 ± 2 °C with a 16-h photoperiod and a light intensity of 25 $\mu mol\, m^{-2} s^{-1}$for 2 weeks in the culture room, and the pots were then transferred to a shade (60 $\mu mol\, m^{-2} s^{-1}$) in the third week. Plantlets were then transferred to glass house in the fourth week.

2.7. Statistical analyses

All the experiments consisted of 25 explants and each was repeated four times unless otherwise stated. Data were statistically analyzed by using either one or two-way analysis of variance (ANOVA). Mean comparisons were made by least significant difference at the 5% probability level. The arcsine square root transformation was applied to proportional data before ANOVA [11].

3. Results and discussion

3.1. Effect of explant types and PGR concentration on callus formation

Effect of explant types and plant growth regulator (PGR) concentration on callus formation and *in vitro* shoot organogenesis of *H. populifolia* were studied in leaf, petiole, and internode explants cultured on MS-basal media as shown in Fig. 1; Fig. 2a and b. The percentage of callus formation and the percentage of explant regenerating shoots varied significantly ($P < 0.001$), depending on the concentration of plant growth regulators (PGR) present in the medium. There was also a significant interaction between the two factors ($P < 0.001$), indicating that the effects of PGR concentration on *in vitro* adventitious shoot regeneration are dependent on explant types. There was no significant difference among the different types of explants in terms of the percentage of callus formation. However, the percentage response of callus formation was significantly increased when IAA was added to the medium in combination with BAP (Table 1). Similarly Rajeswari and Paliwal [26] in *Albizia odoratissima* among the epicotyls, petiole, cotyledon segments tested, the best response in terms of the percentage of shoot regeneration was obtained from epicotyls cultured on MS medium supplemented with 1.5 mg/l BAP, whereas the highest number of shoots per responding explant was recorded on medium containing 0.7 mg/l BAP and 0.1 mg/l NAA. Likewise Gikloo et al. [10] tested the different explants and obtained best result in leaf explants from *Silybum marianum*. In contrast, in *Origanum vulgare*, among the cotyledon, hypocotyls, and root segments tested, cotyledons proved to be the best source for the production of compact and modulated callus [23]. Similarly Gurel et al. [13] reported that hypocotyls and cotyledon explants produced significantly more callus than petiole or leaf explants of *Beta vulgaris*.

The concentration of PGR required inducing a maximum percentage of callus induction varied with the explant type. Internode segments showed the maximum response of 95% callus production when cultured on MS medium supplemented with BA (2.0 mg/l), IAA (0.1 mg/l) and glutamine (25 mg/l), whereas in petiole explants, maximum callus formation of 85% was also observed when cultured on MS medium containing BA (2.0 mg/l), IAA (0.1 mg/l) and glutamine (25 mg/l). Leaf explants required relatively higher concentration of 2 mg BAP in combination with IAA (0.1 mg/l) and glutamine (25 mg/l) for obtaining the maximum response of callus induction (Table 2). Differential responses by different explant types on the same medium may be because of the differences in the endogenous level of plant growth hormone in different explants [25]. Certainly, callus morphology varied among the different explants. Internode-derived callus was green in color, compact in texture, and fast-growing, whereas the callus formed from petiole explants was green white in color, friable in texture, and slow growing. Callus formed from leaf was green in color, friable in texture, and slow-growing. With regard to growth rates, the size of callus after 45 day of culture was greater from internode explants than that of callus formed from either petiole or leaf.

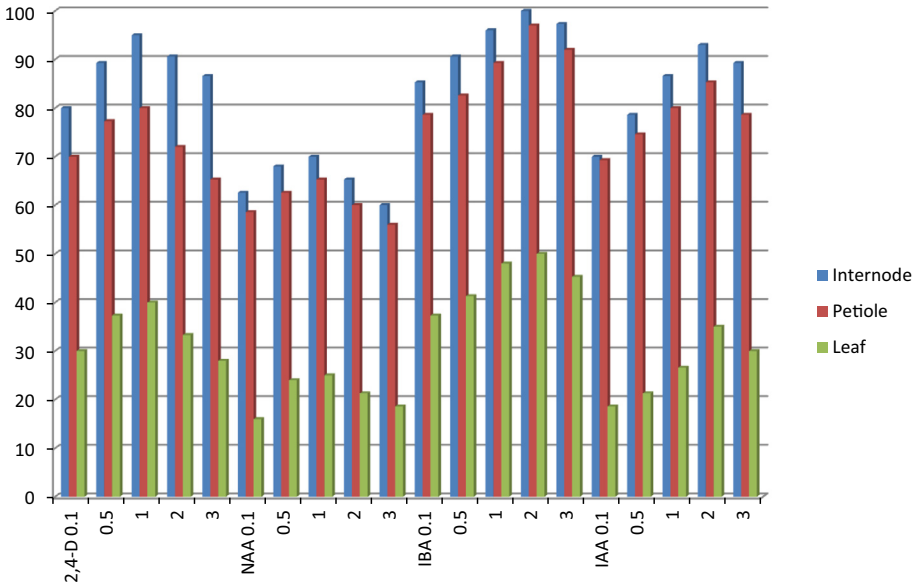

Figure 1 Callus induction from various explants of 45 days old *in vitro* seedlings grown on MS medium supplemented with auxins, after 45 days.

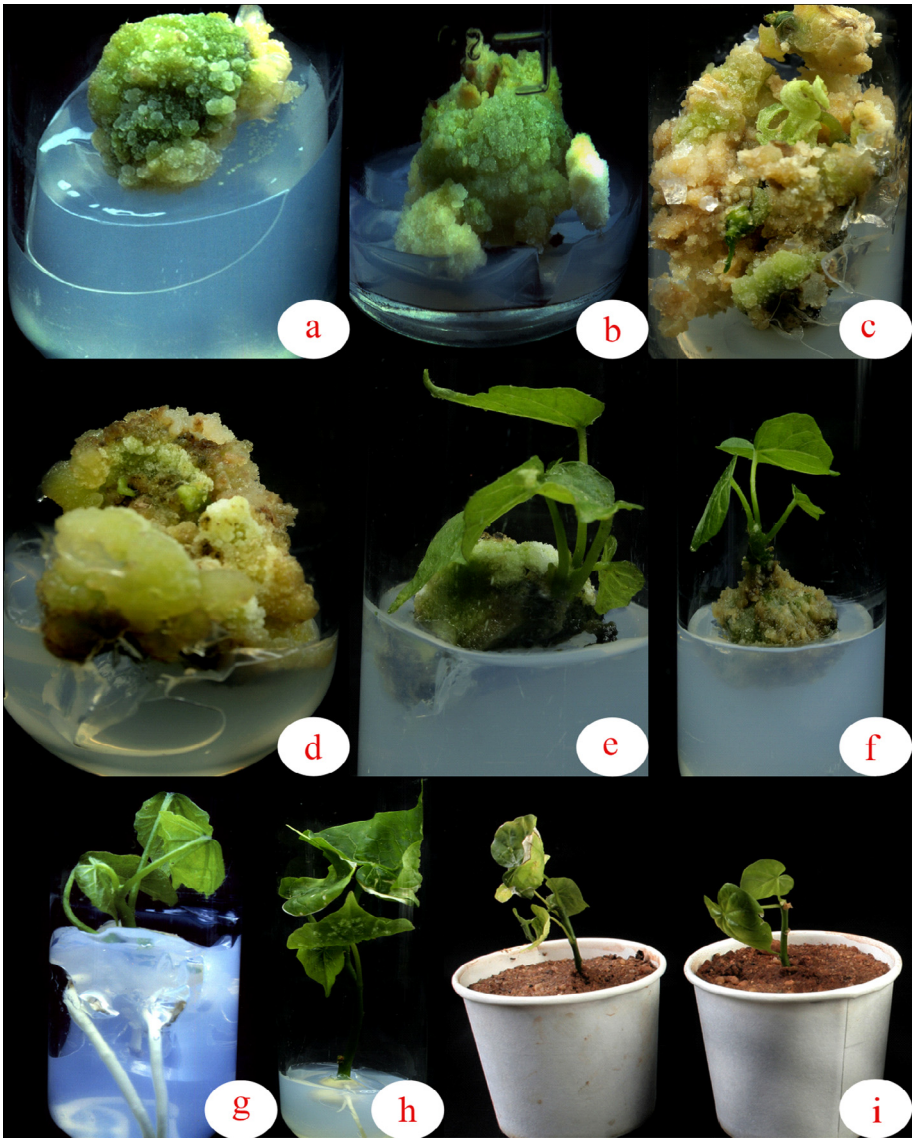

Figure 2 Organogenesis of *Hildegardia populifolia*: Callus induction: (a) internode; (b) petiole. Shoot bud induction: (c) internode; (d) petiole. Shoot regeneration: (e) internode; (f) petiole. Rooting: (g) internode; (h) petiole. (i) hardening.

3.2. Shoot organogenesis from callus

In internode explants, shoot bud differentiation were observed with less callus proliferation and callus and shoot formation occurred simultaneously (Fig. 2c and d). Conversely, in petiole explants, callus formation usually preceded shoot organogenesis. In this study, data on the percentage response of shoot regeneration, shoot number, and percentage of callus formation were recorded after 45 days of culture. Of the different explant types tested, internode segments showed a maximum of 55% shoot regeneration when cultured on MS medium containing 0.5 mg TDZ. However, no significant difference was observed in the percentage of shoot regeneration between internode and petiole explants (Fig 2e and f). Likewise Thakur et al. [31] obtained 75% shoot regeneration frequency in *Populus deltoides* using petiole explants. Leaf explants showed no shoot regeneration under any of the conditions tested in this study. A maximum of 1.8 shoots were regenerated from internode explants which showed a significantly better response for the number of shoots regenerated compared to petiole explants (Table 3). Gubis et al. [12] also reported that hypocotyl and epicotyl segments of *Lycopersicon esculentum* produced the maximum shoot regeneration rate whereas cotyledons showed a poor response on regeneration medium containing 1 mg/l zeatin and 0.1 mg/l IAA. Molina [19] also reported that stems and petioles segments of *Salvia canariensis* produced the maximum shoot regeneration rate whereas stem showed a poor response on regeneration medium contain 4.44 μM BAP and NAA μM. Likewise Martin et al. [18] reported that leaf and internode explants of *Ophiorrhiza prostrata* produced the maximum shoot regeneration in high range of BAP in the MS medium. At high concentration, auxin inhibits the development of the primordial or axillary buds and induces the formation of callus in the presence of activated charcoal in media, the shoots elongated better on media containing lower concentrations of PGRs [6,17]. To promote

Table 1 Callus proliferation from 45 days old *in vitro* seedlings grown on MS medium supplemented with cytokinins and auxins, after 25 days.

Plant growth regulators (mg/l)		Internode		Petiole		Leaf	
		Percentage of response (%)	Nature of callus	Percentage of response (%)	Nature of callus	Percentage of response (%)	Nature of callus
BA							
0.5		76.0^{fg}	GC	70.0^{g}	GC	40.0^{g}	GC
1.0		81.3^{e}	GC	77.3^{ef}	GC	46.6^{e}	GC
2.0		90.0^{c}	GC	85.0^{cd}	GC	50.0^{d}	GC
3.0		84.0^{de}	GC	80.0^{e}	GC	44.0^{ef}	GC
KN							
0.5		54.6^{k}	GFC	40.0^{l}	GFC	28.0^{hi}	GFC
1.0		60.0^{j}	GFC	45.0^{k}	GFC	35.0^{h}	GFC
2.0		50.0^{l}	GFC	37.3^{lm}	GFC	30.0^{h}	GFC
3.0		45.3^{m}	GFC	32.0^{n}	GFC	26.6^{i}	GFC
BA	IAA						
2.0	0.01	85.3^{d}	GCN	80.0^{e}	GCN	50.6^{cd}	GCN
2.0	0.05	93.3^{ab}	GCN	85.3^{c}	GCN	56.0^{c}	GCN
2.0	0.1	95.0^{a}	GCN	90.0^{a}	GCN	65.0^{a}	GCN
2.0	0.5	90.0^{c}	GCN	89.3^{ab}	GCN	60.0^{b}	GCN
BA	IBA						
2.0	0.01	77.3^{f}	GCN	60.0^{ij}	GCN	56.0^{c}	GCN
2.0	0.05	80.0^{ef}	GCN	65.0^{h}	GCN	60.0^{b}	GCN
2.0	0.1	72.0^{h}	GCN	61.3^{i}	GCN	53.3^{cd}	GCN
2.0	0.5	66.6^{i}	GCN	58.6^{j}	GCN	49.3^{de}	GCN

GC – green compact; GFC – green friable callus; GCN – green compact nodular.
Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.

Table 2 Callus proliferation from 45 days old *in vitro* seedlings grown on MS medium supplemented with BA (2.0 mg/l), IAA (0.1 mg/l) and additives, after 25 days.

Additives (mg/l)	Internode		Petiole		Leaf	
	Percentage of response (%)	Nature of callus	Percentage of response (%)	Nature of callus	Percentage of response (%)	Nature of callus
Glutamine						
10	90.0^{b}	GCN	80.0^{b}	GCN	68.0^{ab}	GFC
25	95.0^{a}	GCN	85.0^{a}	GCN	70.0^{a}	GFC
50	89.3^{bc}	GCN	78.6^{bc}	GCN	65.3^{b}	GFC
Sodium citrate						
10	78.6^{de}	GCN	77.3^{c}	GCN	58.6^{cd}	GFC
25	80.0^{d}	GCN	80.0^{b}	GCN	65.3^{b}	GFC
50	74.6^{fg}	GCN	72.0^{d}	GCN	60.0^{c}	GFC
Adenine						
10	65.0^{ij}	GFC	55.0^{fg}	GFC	40.0^{gh}	GFC
25	61.3^{k}	GFC	50.6^{h}	GFC	37.3^{h}	GFC
50	58.6^{kl}	GFC	46.6^{j}	GFC	30.0^{i}	GFC
Adenine sulphate						
10	75.0^{f}	GFC	60.0^{e}	GFC	55.0^{e}	GFC
25	70.0^{h}	GFC	56.0^{f}	GFC	49.3^{f}	GFC
50	66.0^{i}	GFC	49.3^{hi}	GFC	42.6^{g}	GFC

GFC – green friable callus; GCN – green compact nodular.
Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.

shoot elongation and reduce the basal callusing to adding activated charcoal into the medium. The percentage of explants showing axillary bud growth on the culture medium supplement with 10 mg/l activated charcoal can reach to over 70% with an average shoot number (2.5) and nearly 55% with an average shoot length (Table 3). Anuradha and Pullaiah [3] used shoot tip, node and mesocotyl explants for direct shoot regeneration, whereas present experiment leaf, internode and

Table 3 Shoot regeneration response from 45 days old *in vitro* seedlings grown on MS medium supplemented with BA (2.0 mg/l) + IAA (0.1 mg/l) + glutamine (25 mg/l), TDZ and charcoal after 45 days.

TDZ (mg/l)	Activated charcoal (mg/l)	Internode		Petiole	
		Percentage of shoot response (%)	Shoot number	Percentage of shoot response (%)	Shoot number
0.1		52.0^{bc}	1.5^{c}	36.0^{cd}	1.0^{bc}
0.5		55.0^{b}	1.8^{b}	40.0^{b}	1.2^{b}
1.0		49.3^{d}	1.3^{cd}	34.6^{d}	1.0^{c}
2.0		0.0	0.0	0.0	0.0
3.0		0.0	0.0	0.0	0.0
	5	50.0^{c}	1.2^{d}	35.0^{bc}	1.2^{b}
	10	70.0^{a}	2.5^{a}	55.0^{a}	1.8^{a}
	25	55.0^{b}	1.7^{bc}	40.0^{b}	1.0^{bc}

Leaf – no response.
Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.

Table 4 Rooting response of *in vitro* raised shoots from 45 days old *in vitro* seedlings grown on MS medium supplemented with auxins, after 45 days.

Plant growth regulators (mg/l)	Internode			Petiole		
	Rooting response (%)	Root number	Root length (cm)	Rooting response (%)	Root number	Root length (cm)
IBA						
0.1	60.0^{fg}	2.9^{fg}	2.2^{f}	49.3^{e}	2.1^{f}	1.3^{g}
0.5	66.6^{d}	3.1^{f}	2.5^{de}	52.0^{d}	2.6^{d}	1.6^{ef}
1.0	72.0^{c}	3.6^{de}	3.0^{bc}	56.0^{c}	2.9^{bc}	1.9^{cd}
2.0	77.3^{ab}	4.0^{b}	3.1^{b}	61.3^{b}	3.0^{b}	2.5^{b}
3.0	80.0^{a}	4.5^{a}	3.5^{a}	65.0^{a}	3.8^{a}	2.7^{a}
NAA						
0.1	53.3^{i}	2.5^{h}	2.0^{g}	42.6^{g}	1.5^{h}	1.2^{gh}
0.5	61.3^{f}	3.0^{fg}	2.6^{d}	48.0^{ef}	1.8^{g}	1.5^{ef}
1.0	65.0^{de}	3.9^{bc}	2.8^{d}	50.0^{de}	3.0^{b}	2.1^{c}
2.0	60.0^{fg}	3.7^{d}	2.2^{f}	46.6^{f}	2.5^{de}	2.0^{cd}
3.0	56.0^{h}	2.8^{fg}	1.9^{gh}	40.0^{gh}	2.0^{fg}	1.7^{e}

Values are mean of 25 replicates per treatment and repeated thrice. Values with the same superscript are not significantly different at 5% probability level according to DMRT.

petiole explants were used for shoot regeneration through callus formation. Moreover glutamine, sodium citrate, adenine and adenine sulfate was discussed in this experiment.

3.3. Rooting of regenerated shoots

Leafy shoots were transferred to MS basal media containing different concentrations of IBA, NAA and IAA for rooting. 45 day after inoculation, root formation was noticed from the basal cut portion of the shoot. The percentage of shoot forming roots, number of roots per shoot, and length of root were recorded. Rooting method was followed for inducing roots from shoots regenerated from internode and petiole explants in this study. Roots developed on the shoots after 20 days in most of the cultures. However, the data for the percentage of rooting and the number of roots were scored after 45 days of culturing. No significant differences were observed in the rooting responses of microshoots harvested from internodes and petiole explants, but the maximum percentage of rooting (80 ± 5.0), and the maximum number of roots (4.5 ± 0.1) were observed in the shoots obtained from internode explants (Table 4; Fig. 2g and h). MS medium with high concentration of IBA favoured better *in vitro* rooting for internode derived calluogenesis. Likewise Pandey and Sushma [24] obtained more roots using IBA for rooting of *Quercus leucotrichophora*. While IAA showed weak roots with callusing at the base (data not shown). A similar response was also observed by Shriram et al. [30] in *Helicteres isora*.

3.4. Acclimatization

In vitro formed plantlets were transferred to soil, sand and vermiculite mix (2:1:1) and were maintained in culture room under plastic sheets for 25–30 days. Periodical removal of plastic sheets, so as to lower down the high atmospheric humidity gradually, was observed as an effective way for the acclimatization process as more than 85% of the plantlets survived through this. Plantlets were watered with 1/4 – strength MS basal medium devoid of sucrose and meso-inositol at 3 days intervals for a period of 3 weeks. The acclimatization plantlets

were then transferred to pots containing soil and kept under shade for another 8 weeks before transferring to the field. After that, the plantlets were transplanted to soil conditions where more than 95% plantlets were survived. Likewise Chauhan et al. [7] obtained 100% survival in *in vitro* raised plantlet of *Garcinia indica*. The *in vitro* propagated plants developed in the presence of increased moisture; their leaves had cells palisades of which some had great spaces between them and few stomates [4]. Rajeswari and Paliwal [26] obtained the best rate (75%) of survival of the plant *Albizia odoratissima* after acclimatization on vermiculite substrate, while Ndiaye et al. [22] reported 100% of survived plantlets of *Bambusa vulgaris* after acclimatization in pots containing sterile perlite-peat. Rooted plantlets after one month, they were transferred to larger pots containing the same soil and moved to greenhouse. The regenerated plants did not show detectable variation in morphological or growth characteristics compared with the donor plants (Fig. 2i).

4. Conclusion

This report of a protocol for adventitious shoot organogenesis, rooting, and acclimatization of *H. populifolia* presented a first step in developing technologies for the clonal propagation and possible genetic transformation of this species. Of the seedling explants studied, it was found that internode presented a higher potential for shoot organogenesis than petioles and leaf. In addition, the organogenic efficiency of internode explants was affected by the orientation of explants on media and growth regulator conditions. This may realize the demand of fiber industries and minimize the impact of over exploitation of the plants. Besides the propagation of elite cultivars and conservation of this endangered and economical plant, a highly efficient regeneration protocol opens a way for improvement of the plant through genetic transformation strategies.

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